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Award Number: W81XWH-11-1-0051

**TITLE:** Glutamate Signaling and Mitochondrial Dysfunction in Models of Parkinson's Disease

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**REPORT DATE:** March 2014

**TYPE OF REPORT:** Final Option year 2

**PREPARED FOR:** U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

**DISTRIBUTION STATEMENT:** Approved for Public Release;  
Distribution Unlimited

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# REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 0704-0188

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1. REPORT DATE 1 Mar 2014			2. REPORT TYPE Final Option Year 2		3. DATES COVERED 1 Dec 2012-30 Nov 2013	
4. TITLE AND SUBTITLE Glutamate Signaling and Mitochondrial Dysfunction in Models of Parkinson's Disease					5a. CONTRACT NUMBER	
					5b. GRANT NUMBER W81XWH-11-1-0051	
					5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) D. James Surmeier, Ph.D.					5d. PROJECT NUMBER	
Email: j-surmeier@northwestern.edu					5e. TASK NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Northwestern University Chicago, IL 60611					8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012					10. SPONSOR/MONITOR'S ACRONYM(S)	
					11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited						
13. SUPPLEMENTARY NOTES						
14. ABSTRACT  The central hypothesis of the proposal is that in the early stages of PD, an elevation in synaptically released glutamate leads to persistent activation of NMDARs that synergizes with Cav1 calcium channels to significantly increase mitochondrial oxidant stress and neurodegeneration.						
15. SUBJECT TERMS- nothing listed						
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES . 9	19a. NAME OF RESPONSIBLE PERSON USAMRMC	
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)	

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## **1. INTRODUCTION:**

Parkinson's disease (PD) is a widespread, disabling neurodegenerative disorder of unknown etiology. The motor symptoms of PD are triggered by the death of dopaminergic (DA) neurons of the substantia nigra (SNc). Recent work by our group suggests that the selective vulnerability of SNc dopaminergic neurons is attributable to activity-dependent calcium entry through Cav1 channels, resulting in mitochondrial oxidant stress. Although this mechanism provides an explanation for aging related decline in SNc dopaminergic neuron function, it does not explain the accelerated rate of cell loss in the early stages of the disease. **The central hypothesis of the proposal is that in the early stages of PD, an elevation in synaptically released glutamate leads to persistent activation of NMDARs that synergizes with Cav1 calcium channels to significantly increase mitochondrial oxidant stress and neurodegeneration.** Specifically, we hypothesize that increased glutamate release from PPN neurons projecting to the SNc leads to opening of NMDARs and activation of mGluRs, leading to calcium entry and mobilization of intracellular calcium stores that are heavily charged by basal calcium influx during pacemaking. The convergence of Cav1 channel opening and NMDAR/mGluR activation is posited to create a 'calcium storm' that drives mitochondrial OXPHOS, significantly increasing oxidant stress, phenotypic decline and death of dopaminergic neurons. Furthermore, we hypothesize that because the glutamate-mediated stress depends indirectly upon calcium entry during pacemaking, antagonizing the Cav1 calcium channels underlying this influx should significantly reduce this stress and enhance the viability of remaining SNc dopaminergic neurons. Moreover, combining antagonism of Cav1 channels with antagonism of NMDARs and/or mGluRs should prove even more beneficial. We propose to test these hypotheses using a combination of electrophysiological, optical and molecular approaches in brain slices from a novel transgenic mouse model expressing an optical reporter of mitochondrial redox status. **If our hypotheses are correct, it would point to a novel neuroprotective strategy in presymptomatic and symptomatic PD patients with drugs already approved for human use.**

## **2. KEYWORDS:**

Parkinson's disease, calcium, glutamate, NMDA, mitochondrion, oxidant stress, oxidative phosphorylation, neurodegeneration, ion channel, optogenetics, electrophysiology

## **3. OVERALL PROJECT SUMMARY:**

**Specific Aim 1: To characterize interactions between N-methyl-d-aspartate (NMDA) glutamate receptors and Cav1 calcium channels in regulating intracellular calcium and mitochondrial oxidant stress of SNc dopaminergic neurons.**

### **Progress:**

- Our working hypothesis was that dendritically localized NMDA receptor (NMDAR) opening can significantly elevate cytosolic calcium concentration and induce the release of calcium from endoplasmic reticulum (ER) stores, increasing local and global mitochondrial oxidant stress. We have found that antagonizing NMDARs decreases mitochondrial oxidant stress of SNc DA neurons in brain slices. Elevating NMDAR activity by bath application of low micromolar concentrations of NMDA dramatically elevates mitochondrial stress. The chronic antagonism of L-type channels *in vivo* by subcutaneous administration of isradipine (with osmotic minipumps) for 10 days led to a dramatic lowering in mitochondrial oxidant stress in SNc DA neurons. This reduction was accompanied by a reduction in cytosolic calcium oscillations but no change in pacemaking rate.
- We have not found evidence that dendritic NMDAR activation triggers a localized release of calcium from ER stores. A major effort has been made to determine the intrinsic calcium buffering capacity of SNc DA neurons. This is critical to the interpretation of all of our calcium imaging experiments and to our approaches examining calcium-induced-calcium-release. We have developed a new strategy that is based upon published protocols and verified that the intrinsic calcium buffering capacity of SNc DA neurons is very low (below 100). These experiments have been followed up by developing a adenoassociated virus (AAV) vector to deliver a calbindin expression construct to lower intracellular calcium concentrations.

- Our attempts to drive NMDARs using two photon uncaging (2PLU) of glutamate at dendritic sites encountered an experimental problem. Because the location of synapses cannot be readily determined visually in normal brain slices, uncaging glutamate sometimes created large responses and sometimes it did not. We have now developed a mapping strategy with the software controlling our scan head that will allow hot spots (presumed synapses) to be determined quickly. This did not prove reliable. We have now imported mGRASP constructs that will allow us to label presynaptic terminals *in vivo* and to visualize presynaptic terminals with a fluorescent marker in *ex vivo* brain slices. Our effort to develop a mesencephalic cell culture model for this purpose was abandoned. In these model systems, we are testing additional optical probes that will allow us to monitor mitochondrial calcium uptake during synaptic stimulation.
- The NMDA receptors expressed by SNC dopaminergic neurons are widely thought to have GluN2D subunits. Recently, Dr. Steven Traynelis at Emory University has developed a new class of antagonists that specifically target these subunits and those of the GluN2C variety. We have established a collaboration with Dr. Traynelis to test the impact of these compounds on PPN and STN synaptic responses evoked with optogenetic techniques (see below). These compounds are still being tested.
- As described above, we developed an *in vitro* model to study SNC dopaminergic neurons to allow dendritic regions where glutamatergic synapses are formed to be studied more quantitatively. These studies revealed that mitochondrial oxidant stress steadily rose with distance from the cell body. Furthermore, antagonists of L-type channels eliminated differences with somatic mitochondria, suggesting that as the diameter of the dendrite decreases and mitochondrial density falls oxidant stress rises. This helps to understand why dendrites are exquisitely sensitive to stress. In addition, we found that the formation of intracellular inclusions created by extracellular deposition of alpha-synuclein fibrils further increased mitochondrial oxidant stress. However, this oxidant stress was sensitive to treatment with N-acetyl cysteine, suggesting it was coming from the cytosol. Indeed, expression of a novel cytosolic variant of roGFP revealed that cytosolic oxidant stress was dramatically elevated by inclusion formation. This stress is likely to be lysosomal in origin. This provides an important an important clue about the interaction between Cav1.3 channels, NMDA receptors and alpha synuclein in the evolution of PD. It also suggests that strategies aimed at diminishing alpha synuclein aggregation should work in concert with those aimed at diminishing Ca<sup>2+</sup> mediated mitochondrial oxidant stress. Our studies of dendritic oxidant stress and the interaction with synuclein puffs was published in the *Journal of Neuroscience*.
- One of the outgrowths of this work was the discovery that calcium entry into mitochondria of SNC DA neurons increased mitochondrial oxidant stress in large part by stimulating a mitochondrial nitric oxide synthase (NOS). Although the identity of this NOS is uncertain, nitric oxide (NO) production by this mechanism appears to be a general one, as it is present in locus ceruleus (LC) neurons as well. This discovery creates a number of potential therapeutic strategies in PD that complement antagonism of Cav1 channels. This result is included in a manuscript that is now in revision for *Nature Neuroscience*.
- Another key development in the pursuit of this aim was the generation of a new genetically encoded calcium indicator that was targeted to the mitochondrial matrix. This has allowed us for the first time to monitor mitochondrial calcium influx directly in SNC dopaminergic neurons in *ex vivo* brain slices. This is a major advance and promises to give us fundamental new insights.

**Specific Aim 2: To characterize interactions between metabotropic glutamate receptors and Cav1 calcium channels in regulating intracellular calcium and mitochondrial oxidant stress of SNC dopaminergic neurons.**

**Progress:**

- Our working hypothesis is that dendritic metabotropic glutamate receptor type 5 (mGluR5) signaling induces ER calcium release that increases mitochondrial oxidant stress both locally and globally. Furthermore, we hypothesized that diminishing the calcium content of the ER stores by antagonizing Cav1 calcium channels will reduce the mitochondrial stress created by mGluR5 activation. Contrary to our hypothesis, antagonizing mGluR1/5 receptors had no effect on basal mitochondrial oxidant stress in SNC DA neurons in brain slices. Moreover, it had no effect on basal pacemaking or intracellular calcium oscillations. However, blocking IP3 receptors or ryanodine receptors significantly diminished mitochondrial oxidant stress. Moreover, intracellular calcium oscillations appear to be attenuated following ER store depletion. These results suggest that ER stores and calcium-induced calcium release (CICR) are important factors in determining intracellular calcium oscillations and mitochondrial stress but that in the brain slice mGluRs are not active enough to modulate this mechanism. To test this hypothesis, we will apply exogenous mGluR1/5 agonists while monitoring intracellular calcium oscillations and pacemaking. These studies are still ongoing.
- A key part of our current model is that calcium entry through Cav1.3 channels triggers CICR. This CICR process should be amplified by mGluR1/5 stimulation. We also hypothesize that CICR is critical to the influx of calcium into mitochondria - a necessary condition for the elevation of oxidant stress. We have verified this using a blocker of the mitochondrial uniporter (RU360). To better nail this down, we are developing optical probes that will allow us to monitor calcium entry into the mitochondrial matrix as well as calcium exodus from the ER. We are making excellent progress toward this goal and as noted above, viral (AAV2/9) delivery of GCamp6 constructs with mitochondrial targeting sequences have given us excellent expression in SNC dopaminergic neurons *in vivo*. Ex vivo brain slice experiments have shown that these probes are functional.

**Specific Aim 3: To characterize responses of SNC dopaminergic neurons to normal and pathological patterns of activity in glutamatergic fibers arising from the pedunculopontine nucleus (PPN).**

**Progress:**

- Anatomical studies have shown that the principal glutamatergic input to SNC dopaminergic neurons arises from neurons in the PPN. Our working hypothesis is that these inputs will produce responses in SNC dopaminergic neurons similar to those produced by exogenous glutamate application. Our initial strategy was to use optogenetic approaches to study the PPN input to SNC DA neurons. We discovered that because channelrhodopsin2 (ChR2) rapidly desensitizes, burst stimulation of axons was giving us spurious results. As a consequence, we have taken two new approaches. First, we developed a brain slice preparation that preserves the connectivity between the PPN and the SNC, allowing us to electrically stimulate PPN axons and measure synaptic responses in SNC DA neurons. These studies have revealed that 1) the PPN glutamatergic synapse is a high release probability, depressing synapse; 2) there is a robust NMDAR component to the PPN evoked synaptic response; 3) antagonism of ionotropic glutamate receptors essentially eliminates the response to PPN stimulation, suggesting that nicotinic receptors are not prominent mediators of the response to activation of cholinergic neurons in the PPN. Second, we have developed optogenetic tools necessary to selectively activate the cholinergic and glutamatergic inputs to SNC DA neurons using cell-type specific Cre transgenic mice. Although we cannot stimulate these systems at high frequency, we can use this approach to map these inputs onto the dendritic tree of SNC DA neurons and characterize how these inputs are modulated. We also have used this approach to activate these inputs selectively and monitor alterations in pacemaking, calcium oscillations and mitochondrial stress.
- These experiments have yielded surprising results. First, the PPN glutamatergic input to SNC neurons was localized to the proximal 100 microns of the dendritic tree, whereas the subthalamic nucleus (STN) input appears to be more distal. Both inputs had both AMPA and NMDA receptor complements; NMDAR/AMPAR ratios were similar at each, although the

aggregate currents evoked by PPN were larger. The impact of each of these synapses on intracellular  $\text{Ca}^{2+}$  and local mitochondrial oxidant stress is being investigated. Second, the cholinergic input from the PPN evoked smaller, nicotinic acetylcholine receptor (nAChR) synaptic responses. To determine the impact of nicotine on the nAChR responses we bath applied it at physiologically meaningful concentrations (100-500 nM). This dose had no effect on basal pacemaking, but did evoke a small (30-50 pA) inward current. Astonishingly, this inward current was accompanied by a dramatic reduction in the amplitude of intracellular  $\text{Ca}^{2+}$  oscillations attributable to Cav1.3 L-type channels. This effect was not mediated by a direct channel block. As expected from this modulation, mitochondrial oxidant stress was significantly lowered by nicotine. To our knowledge, this provides the first parsimonious explanation for the long known reduction in PD risk that accompanies tobacco smoking. We are currently pursuing the receptor subtype mediating this effect and its mechanism. It is our working hypothesis that the receptor is an alpha6beta2 containing receptor and its effect is mediated by activation of calcineurin. If the former is true, it would point to a therapeutic strategy that lacked abuse potential (alpha4beta2-mediated) or peripheral toxicity (alpha3-mediated).

- We have pursued the observation that nicotine diminished mitochondrial oxidant stress in SNC DA neurons. The first goal was to determine how nicotine was altering intracellular calcium concentration. To this end, we altered our calcium dye from Fluo-4 to Fura-2. Fura-2 has a higher affinity for calcium and better signaling dynamics. Our studies have found that nicotine shifts the mid-point of the intracellular calcium oscillations and reduces the amplitude of the oscillation without changing pacemaking rate or regularity. This effect is mimicked by a positive allosteric modulator of smooth endoplasmic reticulum calcium transporters (SERCAs) and is lost in mice lacking SERCA3, a subtype of SERCA that is expressed at relatively high levels in SNC DA neurons. These mice were generated by Dr. Paul Greengard. These results suggest that nicotine is up-regulating the activity of SERCA3 by a currently unknown mechanism. We also are pursuing the effects of chronic nicotine administration on mitochondrial oxidant stress and mitochondrial density.
- One of the limiting factors in our experiments was our inability to concurrently measure optogenetically evoked responses and mitochondrial oxidant stress. We have overcome this limitation using perforated patch recording from SNC DA neurons in TH-mito-roGFP transgenic mice. These experiments have revealed that repetitive synaptic stimulation produces a transient elevation in local mitochondrial oxidant stress.
- We also are continuing our collaboration with Dr. Traynelis to identify sub-type specific NMDAR antagonists. We are poised to determine if there are differences in the NMDAR complement at STN and PPN synapses.

**Specific Aim 4: To characterize mitochondrial function and glutamatergic signaling of SNC dopaminergic neurons in a model of early stage PD.**

**Progress:**

- Our working hypothesis is that in the early stages of PD, pathological bursting emerges in glutamatergic neurons projecting to the SNC, leading to an elevation in extracellular glutamate and mitochondrial oxidant stress in the remaining dopaminergic neurons.
- The in vitro experiments with partially lesioned animals (using intrastriatal 6-OHDA injections) have proven more difficult than we anticipated. SNC DA neurons in these animals either looked normal in redox status or looked severely compromised. There was no evidence in the healthy neurons of elevated extracellular glutamate. The percentage of cells that looked normal was much lower than the percentage of nominally normal neurons judged by tyrosine hydroxylase (TH) immunocytochemistry. Our interpretation of these studies is that many of the neurons in the SNC of the lesioned animals are very stressed and vulnerable to the added stress of brain slicing, but have normal TH levels. To test this hypothesis, we will give isradipine after establishment of the lesion; animals will be treated for a minimum of one week prior to slicing.

- To complement these studies, acute treatment with MK-801 (an NMDAR antagonist) will be performed.
- In an attempt to take a translational approach, we examined chronic (>1wk) treatment of mice with isradipine (a dihydropyridine that crosses the BBB). As isradipine is moving to a Phase III clinical trial, it is important to determine the effects of chronic treatment. Surprisingly, chronic treatment (with s.c. osmotic minipumps) led to an increase in mitochondrial mass in SNC dopaminergic neurons and a dramatic reduction in oxidant stress. Similar experiments with NMDA receptor antagonists are planned.

**Specific Aim 5: To characterize interactions between glutamate receptors and Cav1 calcium channels in influencing mitochondrial oxidant stress in a genetic model of PD.**

**Progress:**

- These experiments were initiated but encountered difficulty. At ages that are appropriate for these studies, there was significant mitochondrial fragmentation in SNC DA neurons from *DJ-1* or *Sirt3* knockouts. This precluded examination of the interaction between glutamate and Cav1 channels. Our working hypothesis is that this fragmentation is triggered by anoxia/ischemia. In wild-type SNC DA neurons, NMDA receptor activation rapidly induces mitochondrial fragmentation. Our initial attempts to counteract this effect with NMDA receptor antagonists were unsuccessful. However, we learned from Dr. Traynelis that the affinity of the NMDA receptor antagonist we were using (APV) is pH sensitive and drops with the transient ischemia thought to accompany removal of the brain. However, pH-insensitive NMDAR antagonists did not prevent fragmentation.
- An alternative strategy we adopted collaboratively was to determine whether *DJ-1* and *Sirt1/3* deletion alone or in combination increased vulnerability to the toxin MPTP. These studies done with Drs. Charles Meshul and Yungchao Ma have shown that this combination dramatically increases vulnerability of SNC dopaminergic neurons to toxins.
- In an attempt to determine if our model of pathogenesis extended to other cell types, dorsal motor nucleus of the vagus and locus ceruleus neurons from *DJ-1* null mice were examined. In both cell types, mitochondrial oxidant stress was elevated and in both cases it was alleviated by Cav1 channel antagonists.

**4. KEY RESEARCH ACCOMPLISHMENTS:**

- New methods for optically stimulating and characterizing glutamatergic synaptic connections of SNC dopaminergic neurons.
- The subcellular location and physiological properties of these glutamatergic connections have been characterized.
- New methods for monitoring mitochondrial calcium entry have been developed, complementing methods for monitoring redox status in ex vivo brain tissue.
- New methods for examining the relationship between physiological activity and mitochondrial oxidant stress have been developed.
- Discovery that alpha-synuclein inclusions increase cytosolic and mitochondrial oxidant stress in SNC dopaminergic neurons.
- Stimulation of glutamatergic inputs have been found to increase mitochondrial oxidant stress, potentially contributing to pathogenesis in Parkinson's disease.
- Chronic antagonism of Cav1 calcium channels leads to elevated mitochondrial mass and lowered oxidant stress.

- Discovered that stimulation of nicotinic receptors by cholinergic PPN neurons leads to activation of SERCA3 in SNC dopaminergic neurons, lowering intracellular calcium concentration and mitochondrial oxidant stress.

#### **5. CONCLUSIONS:**

Our data are consistent with the core hypothesis of the original proposal: glutamatergic synaptic input is capable of increasing mitochondrial oxidant stress and accelerating the progression of Parkinson's disease.

#### **6. PUBLICATIONS:**

Dryanovski DI, Guzman JN, Xie Z, Galteri DJ, Volpicelli-Daley LA, et al. 2013. Calcium entry and alpha-synuclein inclusions elevate dendritic mitochondrial oxidant stress in dopaminergic neurons. *The Journal of Neuroscience : the official journal of the Society for Neuroscience* 33: 10154-64

Sulzer D, Surmeier DJ. 2013. Neuronal vulnerability, pathogenesis, and Parkinson's disease. *Movement disorders : official journal of the Movement Disorder Society* 28: 715-24

Surmeier DJ, Sulzer D. 2013. The pathology roadmap in Parkinson disease. *Prion* 7: 85-91

Goldberg JA, Guzman JN, Estep CM, Ilijic E, Kondapalli J, et al. 2012. Calcium entry induces mitochondrial oxidant stress in vagal neurons at risk in Parkinson's disease. *Nature neuroscience* 15: 1414-21

Sanchez-Padilla, J., Guzman, J.N., Ilijic, E., Galtieri, D.J., Schieber, S., Oertel, W., Wokosin, D., Schumacker, P. T., Surmeier, D. J. Mitochondrial oxidant stress in locus ceruleus neurons is regulated by activity and nitric oxide synthase (in revision for *Nature Neuroscience*)

#### **Presentations:**

Functional Genomics Symposium, Tübingen, Germany – 4/12

State University of New York, Stony Brook – 4/12

17<sup>th</sup> International Parkinson's Disease Conference – 6/12

Brain Damage and Repair Meeting, Santander, Spain – 7/12

3<sup>rd</sup> World Parkinson's Disease Conference, Montreal, Canada – 9/12

Massachusetts Institute of Technology - 11/12

#### **7. INVENTIONS/PATENTS:**

NONE

#### **8. REPORTABLE OUTCOMES:**

- We have developed a new methodology for measuring neuronal mitochondrial oxidant stress during synaptic stimulation using optogenetic approaches in *ex vivo* brain slices.
- We discovered that nicotine diminishes mitochondrial oxidant stress specifically in SNC dopaminergic neurons that are at risk in Parkinson's disease. The effect appears to be mediated by alpha4/beta2 nicotinic receptors and to involve modulation SERCA3.
- We developed a strategy for mapping the sub-cellular distribution of glutamatergic synapses on the dendrites of SNC dopaminergic neurons using optogenetic approaches.

#### **9. OTHER ACHIEVEMENTS:**

#### **10. REFERENCES:**

NONE

#### **11. APPENDICES:**

NONE